Home cryopreservation of fecal samples: an effective strategy for longitudinal studies using multi-omics approaches

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To the Editor: Accumulating evidence has shown that gut microbiota–host interactions play vital roles in human health and disease. However, the dynamic equilibria between microbes and their hosts limit the utility of single strains or microbial signatures as markers for disease risk prediction and outcome assessment.[1] Analysis of changes in the gut microenvironment using multi-omics approaches has now become routine.[2] In inflammatory bowel disease, and especially during the acute phase, a significant shift in the gut microenvironment occurs within 2 weeks. Therefore, effective strategies to obtain fecal samples every 2 to 3 days are needed. However, challenges in specimen storage, transport, and handling limit long-term sampling ability. The microbial composition of fecal samples stored at room temperature for >24 h is significantly altered. Storage of fecal specimens at −80°C immediately after evacuation is standard procedure but is impractical in real-life situations. An alternative is a storage at −20°C for up to 1 month followed by sample transport and laboratory analysis. The objective of this study was to evaluate whether the gut microenvironment in fecal specimens collected every 2 to 3 days and stored at home at −20°C for up to 1 month could be preserved.

Samples were collected every 3 days in the morning from three healthy volunteers over a period of 1 month. Each sample was transferred to two sterile containers containing stabilization buffer and stored at −80°C or −20°C within 1 h of evacuation. A total of 60 specimens were collected for 16S rRNA gene and internal transcribed spacer 1 region sequencing and 24 specimens were collected for bile acid (BA) analysis. Most samples were collected at 3-day intervals. However, 8 of 60 samples were collected with a delay of >1 day. The quality of fungal RNA used for sequencing was low in 24 of 60 specimens. There were no significant differences in the microbiota, mycobiota, or BA for fecal samples placed at different storage temperatures [Supplementary Figure 1, http://links.lww.com/CM9/B19].

A total of 140 genera were identified in all samples. A median of 74 genera (range: 52–99) was identified per fecal specimen. The 20 most abundant genera represented 74% to 95% of the total microbial community. There were no significant differences in the total numbers of microbial genera (75.0 ± 11.2 *vs.* 74.9 ± 10.3) or in the dominant genera observed at the two storage temperatures. However, fluctuations in the number of microbial genera over short periods of time within individuals with stable dietary habits were observed.

Diversity parameters (alpha and beta diversity) were evaluated to assess time-dependent changes in microbial diversity within individuals and between different storage temperatures. Analysis of alpha diversity, as represented by the Shannon and Simpson indices, indicated that microbial diversity varied among individuals; however, changes in microbial diversity across different time points and storage temperatures were less significant [Figure 1A]. Furthermore, analysis of the most abundant genera in each specimen and principal component analysis based on Bray-Curtis distances revealed that individual differences ($R^2 = 0.68$) explained most of the variance in microbial composition. Of note, variance by time shift in sampling ($R^2 = 0.03$) was higher than that between different storage temperatures ($R^2 < 0.01$) [Figure 1B]. There were significant temporal changes in microbial communities within individuals. However, compared with inter-individual differences, fluctuations resulting from dietary factors and mucosal immune responses were negligible. The dominant gut microbiota at the genus level remained unchanged over time and between different storage temperatures. Drastic alterations in the relative abundance of dominant genera were observed, including a three-fold change in *Bacteroidetes*' relative abundance over time [Figure 1C].

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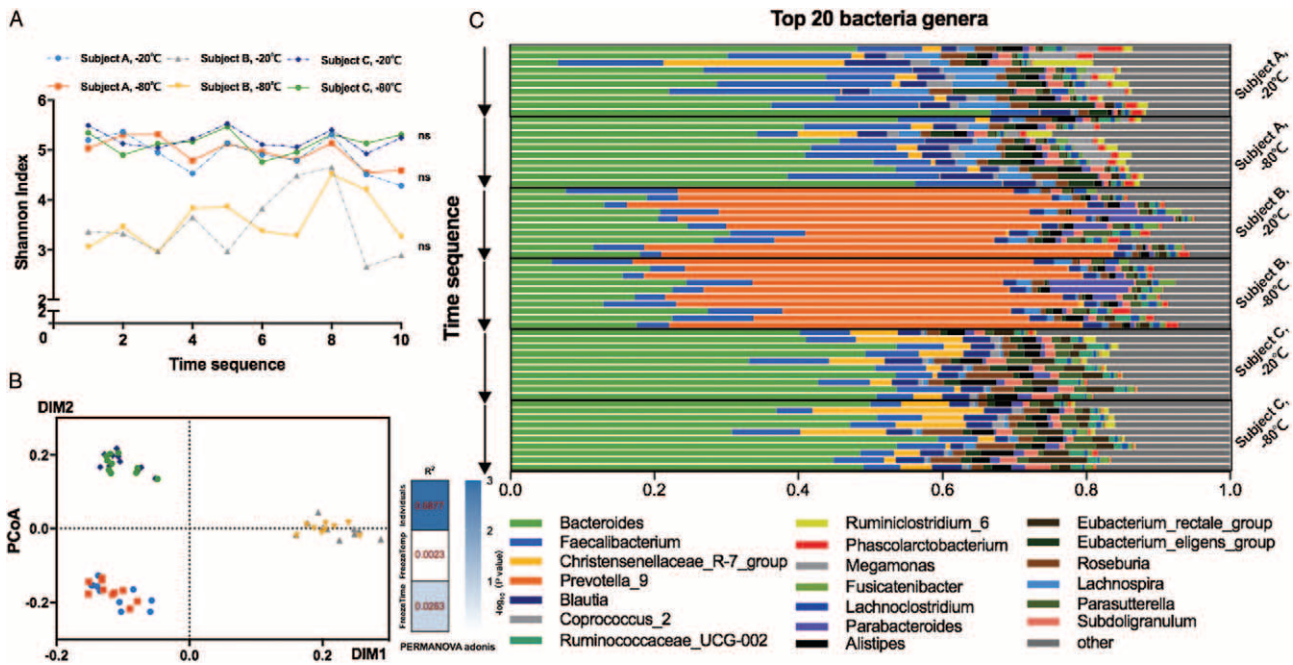

Figure 1: Basic features of bacterial communities. (A) Temporal changes In alpha diversity. Differences between the two storage temperatures were not significantly compared with intra-Individual differences and preservation methods. (B) Principal component analysis plots showing Bray-Curtis dissimilarities between samples. Inter-individual differences accounted for most of the variation in the microbial composition (Bray-Curtis PERMANOVA, $R^2 = 0.69$, $P < 0.001$). (C) Temporal changes in the abundance of the 20 most abundant bacterial genera. PERMANOVA: Permutational multivariate analysis of variance.

Although microbial diversity and abundance were not significantly affected by temperature, extended local similarity analysis (eLSA) was performed to assess dynamic fluctuations in the abundance of bacterial genera during storage. This technique is frequently applied in time-series data analysis to identify temporal patterns of microbial species occurrence. Theoretically, the dynamic alterations in bacterial genera for paired samples should be identical apart from the effects of storage temperature. The changes in the most abundant microbial genera were similar for different storage temperatures (eLSA analysis, $P < 0.05$) [Supplementary Figure 2B, http://links.lww.com/CM9/B19]. However, several dominant genera in the *Bacteroidetes* and *Firmicutes* phyla, including *Bacteroides, Prevotella_9, Faecalibacterium,* and *Blautia,* showed poor local associations compared with other genera [Supplementary Figure 2A, http://links.lww.com/CM9/B19], suggesting that the abundance of these bacteria may have been compromised by storage at −20°C.

A total of 36 samples had high-quality fungal DNA. However, a large portion of unidentified reads per sample was observed (ranging from 0 to 80%). A total of 106 fungal genera were identified among all fecal specimens, with a median of 11.5 (3–27) genera in each sample. Therewereno significant differences in the total numbers of fungal genera (12.8 ± 6.8 *vs.*12.7 ± 6.1) at the two storage temperatures. However, clear temporal changes in the total number of fungal genera within individuals were observed [Supplementary Figure 4C, http://links.lww.com/CM9/B19]. Consistent with these findings, there were significant temporal variations in fungal diversity within individuals reflected by changes in alpha [Supplementary Figure 4A, http://links.lww.com/CM9/B19] and beta diversity [Supplementary Figure 4B, http://links.lww.com/CM9/B19]. Of note, only a small percentage of variation could be explained by inter-individual differences ($R^2 = 0.07$) or intra-individual differences according to Bray-Curtis dissimilarity. Differences associated with time shifts in sampling ($R^2 = 0.05$) could explain part of the variance in mycological composition. Thus, the gut mycobiota was less stable to factors, such as diet and temporal mucosal immune response. Differences in the relative abundance of the top 20 fungal genera over time and between different storage temperatures were analyzed. The dominant genera included *Saccharomyces* (7/36 samples), *Kazachstania* (4/36), *Aspergillus* (4/36), *Cortinarius* (3/36), and *Hygrophorus* (4/36). Within individuals, the dominant genera changed over time: for example, *Saccharomyces,* the dominant genus in most fecal specimens, accounted for 0 to 80.6% of all sequences at different time points [Supplementary Figure 4C, http://links.lww.com/CM9/B19]. The abundance of dominant genera was not significantly affected by the storage temperature ($P > 0.05$), in part because of large intra-individual variation over time. The mycobiota exhibited poor robustness to daily dietary changes, reflected by higher fluctuations in fungal structure over time compared with bacteria. Furthermore, fungal DNA seemed to be less stable for cryopreservation, leading to low DNA quality for next-generation sequencing and sample loss.[3]

Targeted metabolomics was performed for 24 specimens to further assess the effect of storage conditions on fecal

BAs. Forty-nine BAs were identified across all specimens. The primary BA chenodeoxycholic acid and the secondary BAs deoxycholic acid (DCA), isolithocholic acid, lithocholic acid, and ursodeoxycholic acid represented the major BAs, making up 45% to 88% of all BAs. The samples were divided into two groups based on the length of storage. There were no significant differences in the total levels of BAs between the two groups. However, when stratified by length of storage, the levels of nutriacholic acid, alpha-muricholic acid (MCA), beta-MCA, and 3-beta-DCA decreased during long-term storage compared with the gold standard method (Mann-Whitney *U* test, $P < 0.05$). Moreover, levels of several BAs, including hyocholic acid, glycocholic acid, taurolithocholic acid 3-sulfate, and ketolithocholic acid, were decreased at −20°C even in samples stored for short periods [Supplementary Figure 3, http://links.lww.com/CM9/B19]. Few studies have evaluated the effect of storage temperature on the characteristics of metabolites in fecal specimens. Microbial metabolites degrade rapidly at ambient temperature even when using ethanol and other storage methods.[4] The total concentration of BAs was not significantly affected by short-term delayed freezing, with significant changes in approximately one-third of BA subclasses. Storage at −80°C immediately after sample collection is the standard procedure. However, metabolites stored for periods >2 years appeared to be less stable: the relative abundance and concentration of over 50% of serum metabolites were significantly altered.[5] Consistent with these findings, the concentration of several BAs stored at −20°C varied significantly in the samples assessed here. Alternative, convenient, and reliable strategies for fecal BA preservation are required to reduce potential bias in microbiome analysis.

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Conflicts of interest

None.

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